IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Stephen Withers *et al.*

Application No.: 09/837,711

Filed: April 17, 2001

For: Methods and Compositions for the Synthesis of Oligosaccharides Using Mutant Glycosidase Enzymes

Examiner: E. Slobodyansky

Art Unit: 1652

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. The undersigned, a named inventor of the above-identified Application (hereinafter "the Application"), hereby submits the following declaration in support of the patentability of the invention disclosed and claimed in the Application.

2. I have reviewed the pending Office Action dated February 24, 2004, including the remarks and conclusions of the Examiner. In particular, I have reviewed the Examiner's remarks and conclusions relating to how the skilled artisan would interpret the terms "modified glycosyl donor molecule" and "glycoside acceptor molecule." It is my understanding that the Examiner believes that these terms would be interpreted by the skilled artisan to encompass any glycosyl donor and any glycoside acceptor irrespective of the particular glycosynthase being used in the methods as claimed. It is also my understanding that the Examiner believes the specification does not provide sufficient guidance to the skilled artisan to use glycosyl donors other than the respective glycosyl fluoride in the methods as presently claimed.

3. The specification as filed describes, *inter alia*, the production of a glycosynthase by mutating a glycosidase enzyme at one of two catalytically active amino acids

in the active site. It is known in the art that wild-type glycosidase enzymes primarily hydrolyze oligosaccharide products, but also catalyze the reverse reaction and synthesize oligosaccharides to a minor extent. Even when the enzyme is forced to run in the reverse direction the glycosidase reaction can become the primary reaction catalyzed by the glycosidase. It is a well known fundamental principle that any catalyst (including an enzyme) must catalyze the same forward and reverse reaction. In the present invention, the specification teaches a method for achieving a glycosidase enzyme that has lost the ability to catalyze the hydrolysis of oligosaccharides, but which retains the ability to couple a modified glycosyl donor molecule to an acceptor. These modified enzymes have been termed "glycosynthases." Therefore, generally the glycosynthase retains the substrate specificity of the original glycosidase with respect to the "reverse" synthesis reaction.

4. Because the Application as filed is directed to a method for achieving synthesis of a desired oligosaccharide product, and because generally the mutated glycosynthase retains the specificity of the original wild-type glycosidase, thereby providing a means for the skilled artisan to achieve this goal, the skilled artisan, reading the pending claims in light of the specification and as of its effective filing date, would not reasonably interpret the terms "modified glycosyl donor molecule" and "glycoside acceptor molecule" to refer to any random donor-acceptor pair irrespective of the original substrate specificity of the respective wild-type glycosidase enzyme. Instead, the skilled artisan would reasonably interpret these terms in the context of the particular "mutant form of glycosidase enzyme" of interest to the artisan. Many references were available to the skilled artisan prior to the effective filing date of the Application that, for example, characterize the substrate specificity of a large number of glycosidase enzymes in both the forward and reverse direction. See for example, Watt *et al.*, *Currt. Opin. Struct. Biol.* 7:652-660 (1997), a review article published subsequent to the filing date of the present application but which lists a number of references published prior to the filing date.

5. Given a particular glycosidase, the skilled artisan, reading the specification as of the effective filing date of the Application, would further understand that creation of a glycosynthase from a particular glycosidase enzyme, as described in the specification, would provide a glycosynthase with generally the specificity to make the same products that are hydrolyzed by the original glycosidase. As above, it is a fundamental principle that a catalyst

catalyzes the same reaction in the forward and reverse direction. Therefore, the skilled artisan would also know the substrate specificity of the original glycosidase from characterization studies of the substrates hydrolyzed either available in the art or from studies carried out prior to consideration of using the glycosidase as a candidate for mutation to form a glycosynthase. The skilled artisan, given a particular glycosynthase, produced according to the methods described in the specification, would therefore know a variety of corresponding donor-acceptor pair(s) to employ in the claimed methods.

6. The enzymatic reaction catalyzed by a glycosynthase of the invention is a nucleophilic substitution reaction, well understood by the skilled artisan and predictable in the art as of the effective filing date. The skilled artisan, reading the pending claims in light of the specification and as of the effective filing date, would reasonably interpret the term "modified glycosyl donor" as recited in the claims to mean a glycoside molecule modified by a group that functions as a "good leaving group" (*i.e.*, a group that makes the donor more reactive) (*see*, for example, specification at page 4, lines 12-14) and that is "reasonably small" (*i.e.*, small enough to allow the donor molecule to fit within the active site of the particular mutant enzyme) (*see* specification at, *e.g.*, page 12, lines 3-11).

7. Groups that function as a good leaving group in nucleophilic displacement reactions, including those groups recited in the specification, were well-known in the art as of the effective filing date of the Application. (*See, for example,* Mechanism and Theory in Organic Chemistry, 3rd Edition, Lowry and Richardson eds., Addison-Wesley, 1987) Such groups include, for example, azides and formates, as well as those specifically listed in the specification as examples (chlorides, acetates, propionates, and pivaloates, and substituted phenols). Azide and formate were known prior to the effective filing date of the Application to be good nucleophiles and leaving groups (Lowry and Richardson *supra*). Further, it was known to the skilled artisan that azide and formate were smaller or of similar size to certain examples of leaving groups provided in the specification. Still further, prior to the effective filing date of the Application, azide and formate were known to be substrates of a mutant glycosidase enzyme (*Agrobacterium faecalis* β-glucosidase) in a rescue reaction. See, for example, Wang *et al.*, *J. Am. Chem. Soc.* 116:11594-11595 (1994); cited previously during prosecution). Wang *et al.* (*supra*) also provide an average distance between each pair of active site carboxylate oxygen

atoms for a number of glycosidase enzymes. Provided with the guidance provided in the specification alone or in combination with the information available in the art prior to the effective filing date of the Application it is well within the skill of the artisan to select a leaving group of the proper size.

8. Further, in order to address continued concerns of the Examiner relating to the scope of alternative glycosyl donors we have recently carried out additional experiments described herein that demonstrate the use of an alternative glycosyl donor other than a glycosyl fluoride in transglycosylation reactions catalyzed by a mutant glycosidase of the present invention. The study has been carried out under my direction and control and are part of ongoing studies into the characteristics of mutant enzymes as described in the present application. The following summarizes an experiment using α-glucosyl azide as an alternate donor to α-glycosyl fluoride with a mutant β-glycosidase. The α-glucosyl azide donor used in the experiment is not specifically listed as an example in the specification, but as above, would have been considered by a skilled artisan to be a glycosyl donor that is modified by a group that is "reasonably small and which function[s] as relatively good leaving group[]" as defined in the specification at, for example page 4, lines 11-13 and page 12, lines 3 through 11.

9. The α-glucosyl azide was synthesized by azide reaction with per-O-acetylated β-glucosyl chloride to give the protected α-glucosyl azide. The protected α-glucosyl azide was then deprotected with sodium methoxide in methanol. This purified and characterized substrate was used in the enzymatic study described below.

10. In the glycosynthase reactions, the modified α-glucosyl azide donor produced as described in paragraph 9, *supra*, was transglycosylated onto the glycoside acceptor *p*NP β-D-glucoside. The α-glucosyl azide donor (25 mM or 100 mM) was incubated in the presence of *p*NP β-D-glucoside (12.5 or 25 mM, respectively) with the nucleophile mutant *Agrobacterium* E358G β-glucosidase (AbgE358G) at 5.0 mg/ml. 25 mM α-glucosyl azide and 12.5 mM *p*NP β-D-glucoside were also tested using AbgE358G at 2.5 mg/ml.

11. Thin layer chromatograpy (TLC) analysis demonstrated that, using the α-glucosyl azide donor, a transglycosylation reaction occurred as indicated by the formation of new compounds that are both UV-active and stained by sulfuric acid. Two images of each TLC plate

are shown in Exhibit 1, one showing the UV lamp image (which shows aromatic containing species) and the other showing the result of staining with sulfuric acid (which stains for anything containing a sugar). The two TLCs on the left show the reaction with 25 mM glucosyl azide donor and 12.5 mM *p*NP β-D-glucoside acceptor. The TLCs on the right show the reaction with 100 mM glucosyl azide and 25 mM *p*NP β-D-glucoside. These data clearly show that the AbgE358G mutant will form a disaccharide (and a small amount of trisaccharide) under these conditions.

12. Based on (1) the specification as filed, including the specification's disclosure as reflected in paragraph 3, *supra*; (2) the knowledge in the art as of the effective filing date of the Application, including the knowledge of the skilled artisan as reflected in paragraphs 4 through 7, *supra*; and (3) the current studies described in paragraphs 8 through 11, *supra*, as well as in the previously submitted Declaration under 37 C.F.R. § 1.132, filed November 24, 2003, I conclude that modified glycosyl donors as defined in the specification, including, for example, glycosyl azides, glycosyl formates, and those specifically listed in the specification (*e.g.*, glycosyl chlorides, acetates, propionates, and pivaloates), can serve as a modified glycosyl donor molecule in the same manner as the glycosyl fluorides specifically exemplified in the present application when used with a mutant glycosidase of the present invention.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize validity of the application or any patent issuing thereon.

Date: 24th August 2004 By: SGW
Stephen G. Withers

Enzyme-catalyzed formation of glycosidic linkages

Gregory M Watt, Philip AS Lowden and Sabine L Flitsch*

Significant progress has recently been achieved in the use of glycosidases and glycosyltransferases as synthetic tools. Glycosidases have been used to synthesize trisaccharides with a reasonable overall yield, as well as high-mannose neoglycoconjugates. Studies on glycosyltransferases have defined reaction mechanisms and demonstrated reasonable substrate tolerance of these enzymes. Effective methodology for the synthesis of defined glycoproteins has also been demonstrated.

Addresses
The Edinburgh Centre for Protein Technology, Department of Chemistry, King's Buildings, The University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, UK
*e-mail: s.flitsch@ed.ac.uk

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Abbreviations

Endo-A	*endo*-β-*N*-acetylglucosaminidase
FucT	α-1,3-fucosyltransferase
GalNAc	*N*-acetyl-galactosamine
GalT	β-1,4-galactosyltransferase
GlcNAc	*N*-acetyl-glucosamine
Man	mannose
OST	oligosaccharyltransferase
SialT	α-2,3-sialyltransferase
Teoc	trichloroethyl carbamate

Introduction

The selective formation of glycosidic linkages is the most challenging step in the synthesis of oligosaccharides, glycolipids, proteoglycans, glycopeptides and glycoproteins. Although the formation of glycosidic linkages can be achieved with both regio- and stereoselectivity using chemical synthesis [1], it remains a long and low-yield process, which is even more difficult for glycoconjugate synthesis. Enzymes have therefore been explored as catalysts for the formation of glycosidic linkages, because they dispense with the need for complex protecting-group strategies and can give access to glycoconjugates, and even complex glycoproteins [2,3]. Whilst the principle of using enzymes in saccharide synthesis has been well established, the range of glycosides that can be formed by enzyme catalysis is still limited. Research has therefore focused on expanding the repertoire of enzyme-catalyzed reactions by investigating the substrate tolerance of enzymes using substrate analogues, and by trying to isolate new enzyme activities.

For the synthesis of glycosidic linkages, both the biosynthetic glycosyltransferases and the hydrolytic glycosidases can be used. The latter have to be employed under 'non-natural' conditions because hydrolysis is normally favoured. The past four years have seen significant advances in the use of these enzymes. We review some of the most interesting examples.

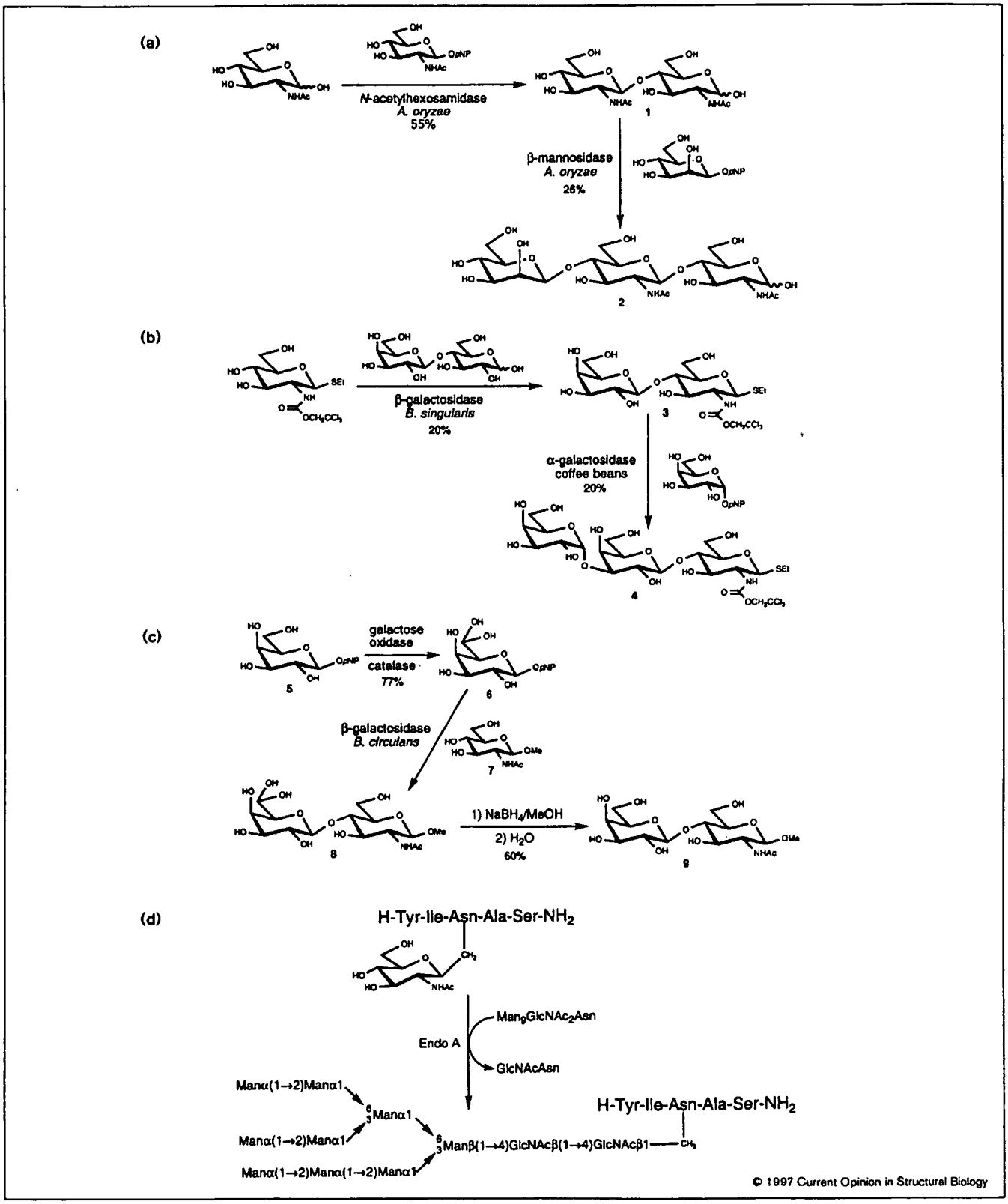
Glycosidases

Glycosidases are abundant in nature, where they cleave glycosidic linkages. Given the right *in vitro* reaction conditions, however, glycosidases can also be used for the reverse reaction, that is, the formation of glycosidic linkages. The thermodynamically favourable hydrolysis in water can be overcome by using two methods: first, by running the reaction under kinetically controlled conditions by using a good glycosyl donor substrate, such as an aryl glycoside or a cheaply available disaccharide; second, the glycosidase-catalyzed reactions can be run with a large excess of substrate (molar concentrations) or, in some cases, in organic solvents, such that glycoside formation becomes thermodynamically favourable. Many acceptor substrates bind preferentially to the acceptor site of the enzyme, thus encouraging glycoside formation over hydrolysis of the substrates. Although glycosidic hydrolysis can be reduced, yields for the synthesis of glycosidic linkages are generally low, which restricts this method to the preparation of small oligosaccharides.

Glycosidases are stereospecific, that is, they catalyze the formation of either the α- or the β-glycosidic linkage, and they are specific for the glycosyl donor substrate. They can generate a number of regioisomers, however, depending on the acceptor structure. In the early experiments, mainly 1,6-linked products in mixtures with smaller amounts of 1,2- 1,3- or 1,4-linked saccharides were found to be the glycosylation products; however, more recently, greater selectivity has been achieved by careful choice of substrate and enzyme. For example, Crout and coworkers [4] have shown that using the kinetic glycosylation method with *N*-acetylhexosaminidase (Figure 1a), 1,4-linked saccharides such as **1** are preferentially formed, with some 1,6-linked disaccharides as side products. The disaccharide **1** has been used in turn as an acceptor substrate for the β-mannosidase of the same organism to generate the *N*-glycan core trisaccharide **2** [5•]. The result is interesting because the 4-hydroxyl group is generally considered the most unreactive in chemical glycosylations [6].

Another recent example by Nilsson [7•] is the two-step synthesis of the trisaccharide **4** (Figure 1b), which has potential applications as an inhibitor of the hyperacute rejection process. Both the disaccharide **3** and trisaccharide **4** have been obtained in 20% yield on a preparative scale using a β-galactosidase and an α-galactosidase successively. The unnatural trichloroethyl carbamate (Teoc) protecting group on the 2-amino group is well tolerated by the

Figure 1



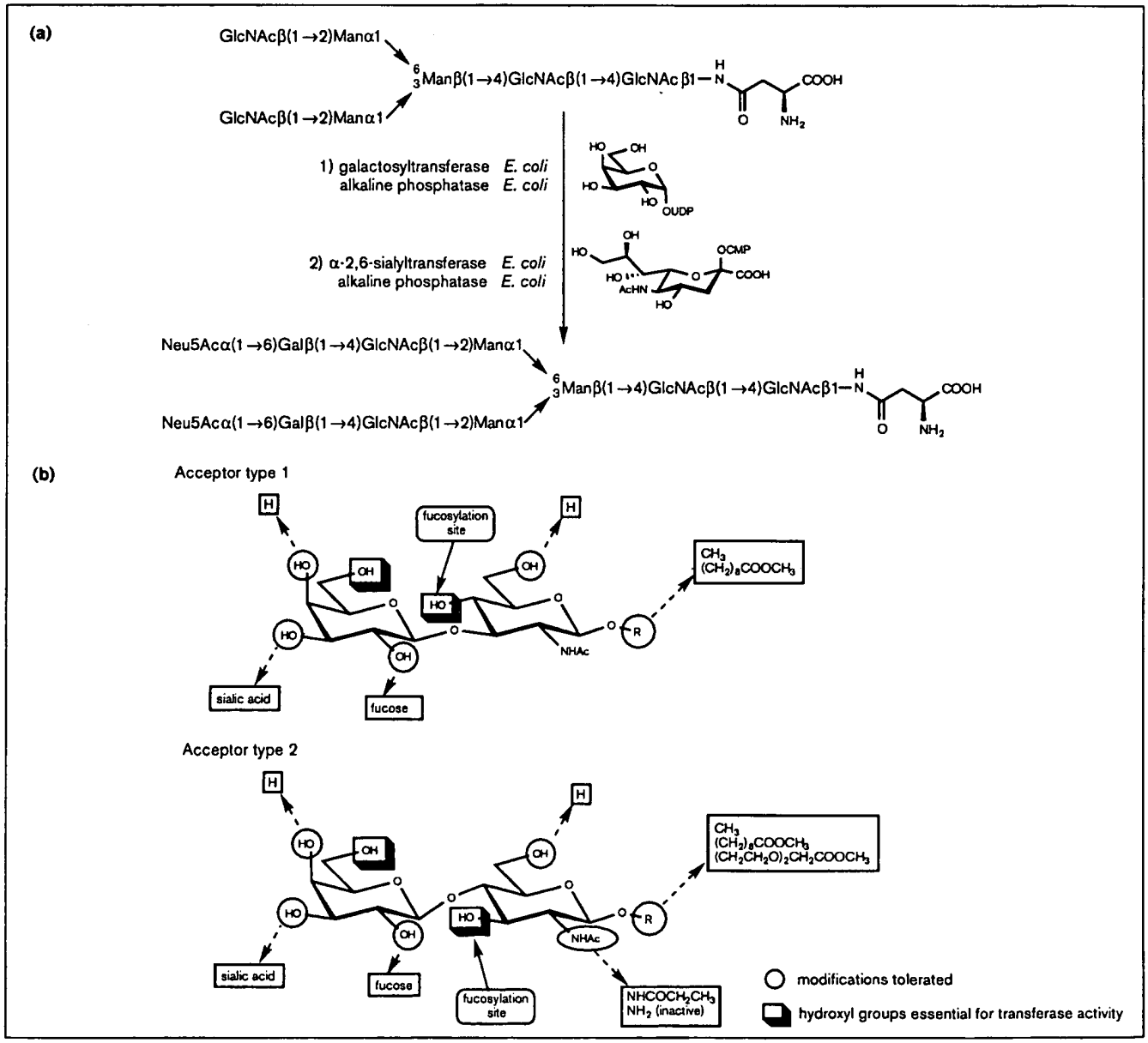
Examples of the use of glycosidases in synthesis. **(a)** Synthesis of the core trisaccharide of *N*-linked glycans by sequential use of *N*-acetylhexosaminidase and β-mannosidase [5•]. **(b)** Trisaccharide synthesis, incorporating the unnatural Teoc protecting group [7•]. **(c)** The yield of a glycosidase-catalyzed glycosylation is increased by prior oxidation of the 6 position of the donor [9]. **(d)** Synthesis of a high-mannose neoglycoconjugate using the Endo-A from *Arthrobacter protophormiae* [16••].

enzymes, demonstrating that analogues can be prepared by this method. A similar synthesis of a trisaccharide in which the natural *N*-acetyl group is used in place of the *N*-Teoc group has also been reported [8].

The yield of a glycosidase-catalyzed galactosylation has been improved by oxidising the donor substrate 5 prior to transfer [9] (Figure 1c). The 6-*O*-hydrate 6 is still accepted by the galactosidase and couples with the methyl glycoside acceptor 7, but the resistance of the resulting disaccharide 8 to competing glycosidase hydrolysis is markedly higher than that of 9. The yield of the disaccharide 9 obtained after sodium borohydride reduction is 60%, which is double the yield obtained using the unoxidized donor 5.

Very little work has been reported on the synthesis of glycosides of simple alcohols using glycosidases. Crout and coworkers [10] have effectively prepared glycosides of alcohols under thermodynamic conditions using high concentrations of the alcohol as the solvent. Recently,

Figure 2



Examples of the use of glycosyltransferases in synthesis. **(a)** Chemo-enzymatic synthesis of an asparagine-linked undecasaccharide using glycosyltransferases [18•]. **(b)** Summary of the glycosyl acceptor modifications tolerated by the α-1,3- and the α-1,3/4-fucosyltransferases from human milk. Type I acceptors contain the 1,3-glycosidic linkage, whereas type II acceptors contain the 1,4-glycosidic linkage. Adapted with permission from [23•].

heating using microwave technology [11,12] has been shown to improve yields, but this is restricted to thermostable enzymes.

The natural function of *endo*-β-*N*-acetylglucosaminidase (Endo-A) is the cleavage of the glycosidic bond in the *N,N'*-diacetylchitobiose moiety of high mannose type *N*-glycans. To reduce the hydrolytic nature of the enzyme, Lee and coworkers [13] have used organic solvents in the reaction media, thereby significantly increasing the transglycosylation activity of Endo-A. Acceptors based on glucose, mannose, 2-deoxyglucose [13] and fucose [14] are also accepted, as well as simple alcohols and a wide range of GlcNAc glycosides [15]. This procedure has been successfully applied to the chemoenzymatic synthesis of a high-mannose-type *N*-glycopeptide analogue (Figure 1d) [16••].

Glycosyltransferases

Glycosyltransferases are involved in the biosynthesis of oligosaccharides; they catalyze the transfer of a specific monosaccharide from a nucleotide diphosphate donor to a specific glycosyl acceptor. The application of glycosyltransferases to the preparative synthesis of oligosaccharides is restricted because of their rarity and the requirement for expensive nucleotide donors. Only five transferases are commercially available, namely, β-1,4-galactosyltransferase, α-2,3-sialyltransferase, α-2,6-sialyltransferase, α1-,2-mannosyltransferase and α-1,3-fucosyltransferase V; however, cloning and overexpression techniques have greatly increased the number of glycosyltransferases that can be used for preparative synthesis of oligosaccharides [3]. Other transferases that can be readily isolated from tissue sources and used for preparative synthesis on a milligram scale have been tabulated in a recent review [17].

Because glycosyltransferases are high yielding and essentially regio- and stereoselective, their use for preparative oligosaccharide synthesis is very attractive, in particular for the synthesis of large glycan structures. A recent example is shown in Figure 2a, in which a heptasaccharide asparagine conjugate is in turn galactosylated and sialylated, using galactosyltransferase and α-2,6-sialyltransferase, respectively, in the presence of alkaline phosphatase to afford a sialylated undecasaccharide in 86% yield [18•].

Mechanistic and substrate specificity studies have been carried out on human α-1,3-fucosyltransferase V by Wong and coworkers [19]. Evidence for a general base mechanism is supported by a pH-rate profile and proton inventory studies have indicated that a single proton transfer occurs in the transition state. Further work, involving kinetic isotope studies with GDP-[1-^{2}H]-fucose and inhibitory studies with GDP-(2-deoxy-2-fluoro)-fucose, supports evidence for a charged sp^2-hybridized transition state, in which glycosidic cleavage occurs prior to nucleophilic attack [20]. A new chemical synthesis of sugar nucleotides has been applied to the synthesis of the GDP-fucose derivatives [21].

Palcic, Bundle and coworkers [22•] have successfully overexpressed in *Escherichia coli*, purified and characterized the α-1,4-GalNAc-transferase that synthesizes the blood group A antigen. They have also prepared mutants in which 1–3 amino acids are changed to those present at the homologous positions in the sequence of the α-1,4-Gal-transferase that uses the same acceptor to synthesize the blood group B antigen. Three substitutions are required to change the donor specificity, whereas a single substitution actually increases the activity towards UDP-GalNAc. The recombinant enzymes have been used in the preparative-scale synthesis of the blood group A and B antigens.

In work directed towards the search for new glycosyltransferase activities, Gosselin and Palcic [23•] have isolated α-1,3- and α-1,3/4-fucosyltransferases from human milk, in order to map their acceptor-binding sites. Kinetic studies using monodeoxygenated derivatives of acceptor substrates have been carried out, and it has been shown that modifications are tolerated at every hydoxyl group except for the 6-hydroxyl of galactose and the fucosylation sites themselves (Figure 2b). These results are analogous with those for cloned fucosyltransferases III–V [24].

Studies on the substrate specificity of galactosyltransferase on glucosamine derivatives [25] have shown that the enzyme tolerates replacements of the natural *N*-acetyl substituent with a variety of groups, including charged and sulphonamide groups. The same replacements are tolerated by α-2,3-sialyltransferase [26] and fucosyltransferase [27], to afford a range of sialyl Lewis^x derivatives. Other examples of galactosyltransferase accepting unnatural substrates have been published [28,29]. Flexibility with regard to glycosyl donor has been demonstrated for the human milk α-1,3/4-fucosyltransferase. Carbon backbone elongated derivatives of GDP-fucose have been synthesized and used as substrates for glycosylation of Gal-β-1,4-GlcNAc [30].

The use of multienzyme systems for the *in situ* regeneration of sugar nucleotides in glycosyltransferase-catalyzed synthesis of oligosaccharides [2,31] solves not only the problem of obtaining expensive sugar nucleotides but also the inhibitory effects of cleaved diphosphate nucleotides. In a one-pot synthesis described by Kren and Thiem [32], a galactosidase-catalyzed synthesis of β-Gal-1,3-GalNAc is coupled with an α-2,3-sialyltransferase multienzyme system that regenerates CMP-Neu5Ac to afford sialyl T-antigen in 36% yield. In work described by Ichikawa *et al.* [33], the sucrose phosphorylase reaction is incorporated into a galactosyltransferase multienzyme system that regenerates UDP-galactose. This allows the *in situ* generation of glucose-1-phosphate — an essential component of this process.

Solid-phase synthesis using glycosyltransferases

The advantages of solid-phase synthesis are well known, especially in the fields of peptide and nucleic acid chemistry. They include the ease of purification and the potential for the construction of combinatorial libraries. The development of solid-phase oligosaccharide synthesis has been relatively slow, however, because of the inherent difficulties of regio- and stereocontrol in the formation of glycosidic linkages.

Solid-phase synthesis of oligosaccharides and glycopeptides using glycosyltransferases was introduced in 1994 by Wong, Paulson and coworkers [34]. They prepared a glycopeptide containing sialyl Lewis^x by enzymatic glycosylation of a chemically synthesized glycopeptide attached to silica (Figure 3). Further reports have been made of enzymatic glycosylation using different supports—controlled pore glass [35], polyethylene glycol-polyacrylamide copolymer [36] and polyacrylamide [37]. Soluble polyacrylamide supports have also been utilized [38–40]. Various methods have been tested for cleavage of oligosaccharide from the support, including chymotrypsin [34,39], hydrazinolysis [35], acid [36], hydrogenation [38], photolysis [37,40] and reductive cleavage of disulphides [41].

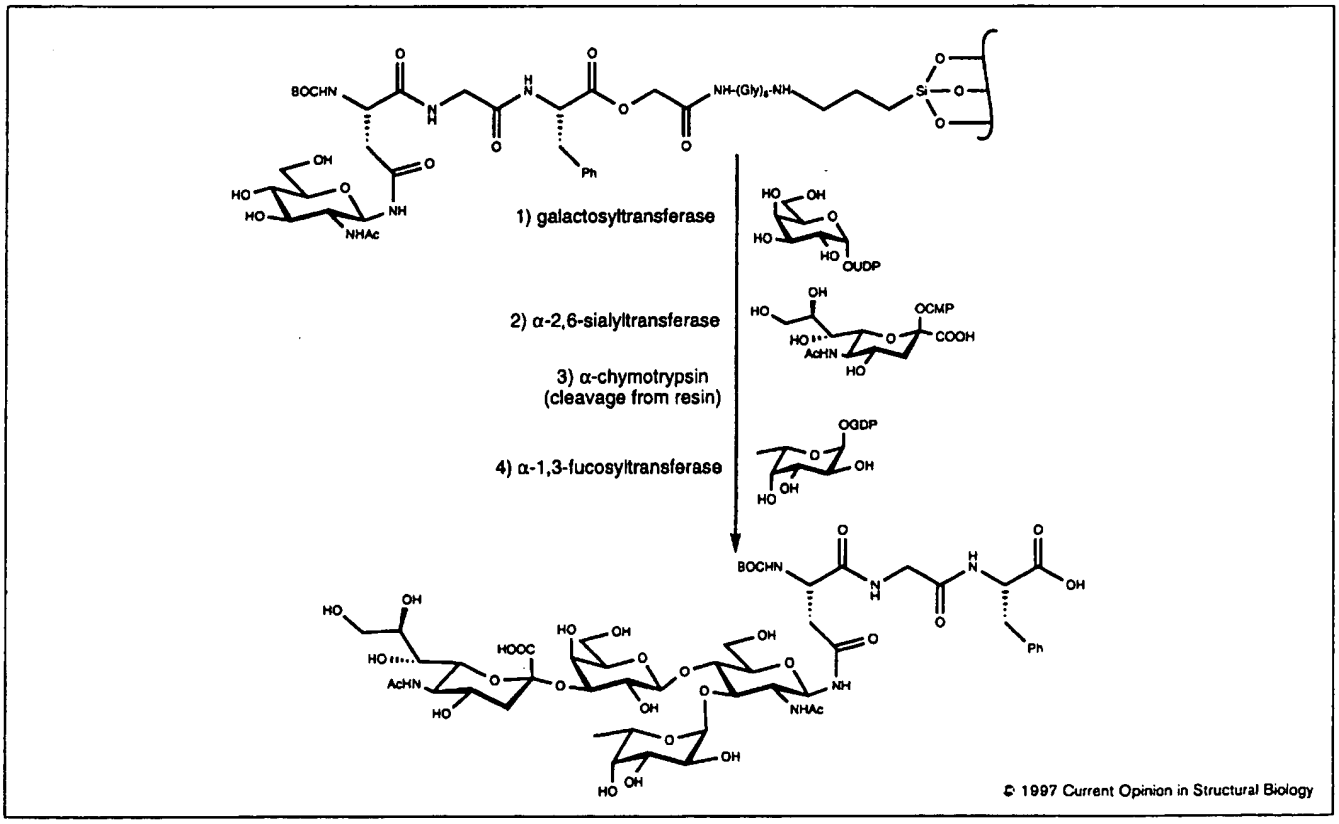
Oligosaccharyltransferase

One of the principle goals of research into oligosaccharide synthesis is the synthesis of homogeneous glycoproteins. This synthesis would allow a more precise definition of the roles of individual glycans in determining the physical properties and the biological function of their parent glycoproteins [42].

An obvious approach would be to use the enzymes that perform the glycosylation of proteins *in vivo*. *N*-linked glycosylation is performed by the enzyme oligosaccharyltransferase (OST), which catalyzes the transglycosylation of a preformed tetradecasaccharide from dolichylpyrophosphate to an asparagine residue on a nascent polypeptide [43,44•]. OST has been purified, and its peptide sequence has been determined from various sources. One can envisage the use of OST to transfer chemically synthesized oligosaccharides onto proteins. Recent work [45,46] has defined limits for the substrate specificity of the OST.

Mechanistic studies have identified the minimal acceptor to be the tripeptide RCONH-Asn-X-Ser/Thr-CONHR, where X is any amino acid except proline, although it has also been shown that *threo*-α,β-diamino-*n*-butyrate can be accepted in place of the serine or threonine [45].

Figure 3



Use of glycosyltransferase in the solid-phase synthesis of a sialyl Lewis^x glycopeptide [34].

The carbohydrate moiety must contain at least the disaccharide chitobiose ($GlcNAc_2$), although transfer of larger oligosaccharides is faster. OST seems to be less flexible, however, with regard to the lipid moiety. The activity of yeast OST has been measured for various analogues of dolichylpyrophosphorylchitobiose containing shorter lipids (Figure 4), but none of the analogues result in significant levels of glycosylation [46]. This is unfortunate, as it would be advantageous to use shorter and, hence, more stable and easily synthesized sugar donors, as has been achieved for the yeast β-1,4-mannosyltransferase [47], which can utilize phytanylpyrophosphorylchitobiose as acceptor.

Synthesis of glycoproteins

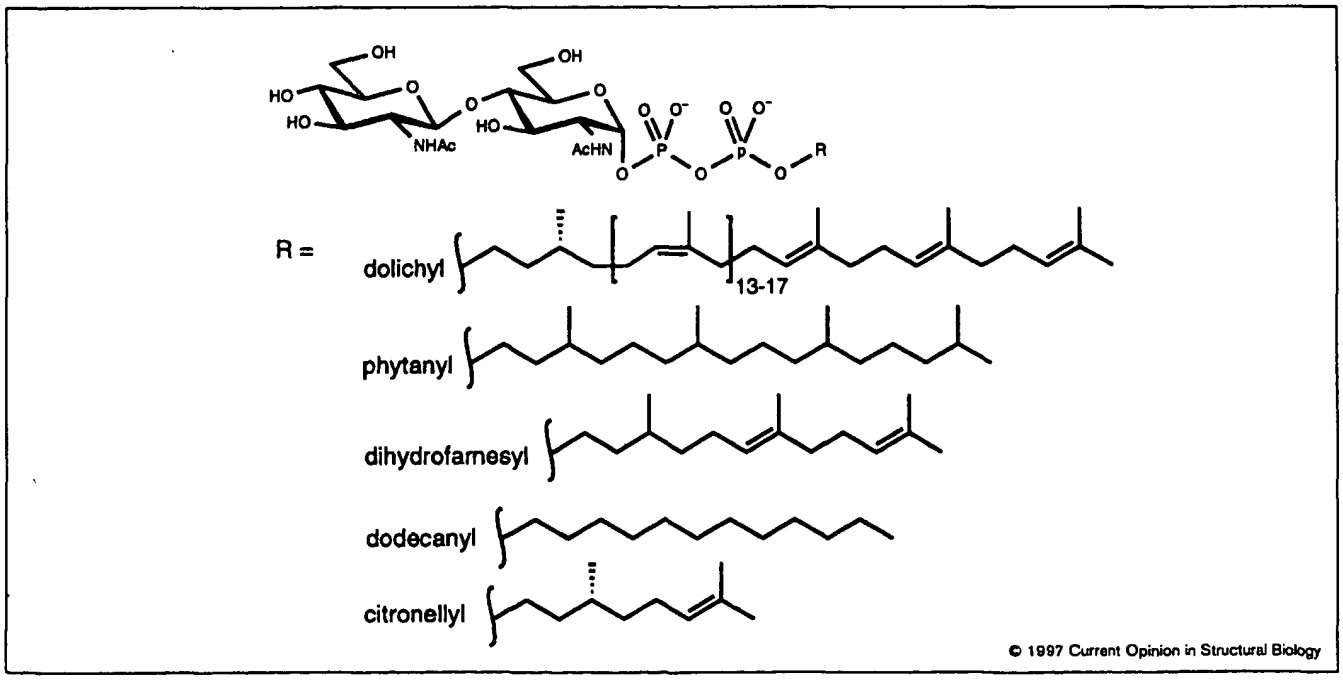
A recent breakthrough has brought us a big step closer to the total synthesis of glycoproteins. Wong and coworkers [48••] have reported the synthesis of an unnatural glycoform of ribonuclease using two different strategies (Figure 5). Ribonuclease B contains a single *N*-linked glycosylation site and exists as a series of high-mannose glycoforms. Treatment with endoglycosidase H gives a ribonuclease derivative, GlcNAc-RNase, with a single GlcNAc attached at this site. Treatment with β-1,4-galactosyltransferase (GalT) and a cofactor recycling system gives rise to the disaccharide-linked protein in 76% yield. Further treatment with α-1,3-fucosyltransferase V (FucT) or α-2,3-sialyltransferase (SialT), or SialT followed by FucT gives rise to the predicted glycoprotein products in yields of 72%, 85% and 74% (for the FucT-catalyzed step), respectively. The product of the three-step synthesis is a protein-bound form of sialyl Lewis^x. In an extension of this approach, Wong and coworkers [48••] have treated the GlcNAc-RNase with subtilisin BPN′ to give two fragments consisting of residues 1–20 and 21–124 (with GlcNAc attached). These peptides are religated using subtilisin 8397 in glycerol to give full length GlcNAc-RNase. This work is exciting because it shows that glycosyltransferases can effectively recognize unnatural glycoprotein substrates to provide designed protein-linked glycans. It also demonstrates the feasibility of ligating synthetic glycopeptides to give full-length glycoproteins.

Another recent report holds promise for the synthesis of glycoproteins. Hecht and coworkers [49••] have reported the first incorporation of a glycosyl-amino acid into a protein using the method of unnatural amino acid mutagenesis [50]. Glucosyl-serine has been incorporated into firefly luciferase by *in vitro* translation in the presence of a synthetic tRNA aminoacylated with glucosyl-serine.

Whole cell methods

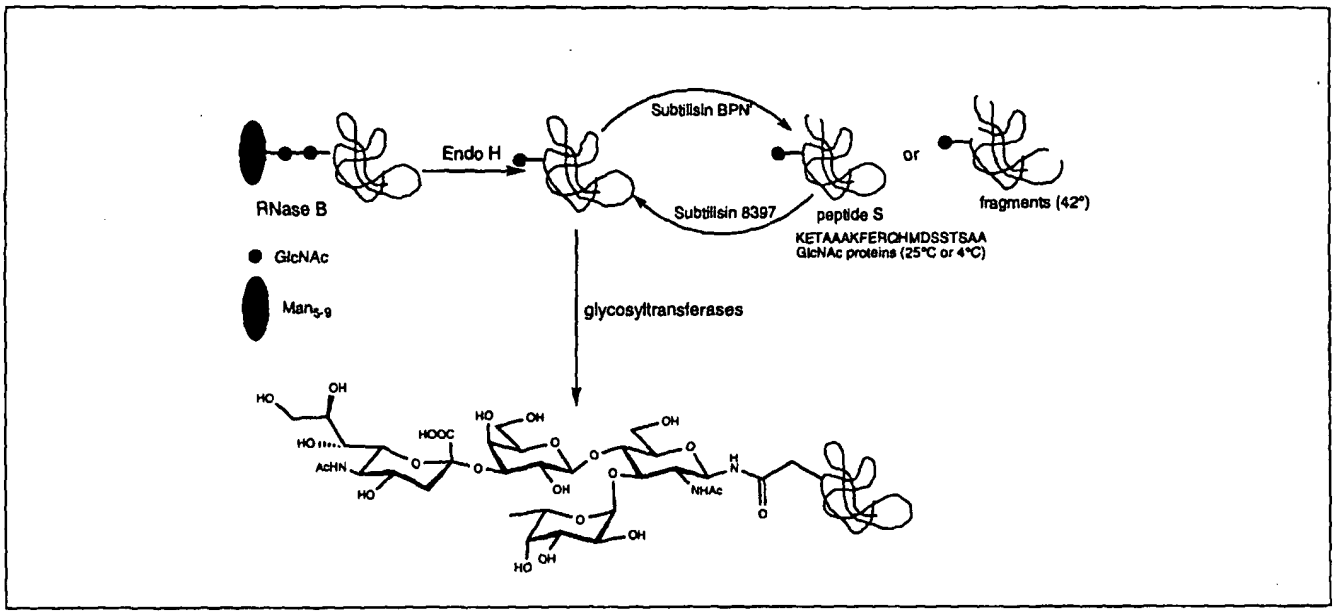
Although great progress has been made in the use of purified glycosyltransferases, this methodology still suffers from the difficulty of purifying these enzymes. The use of whole cell biotransformation would be an attractive alternative for the large-scale production of oligosaccharides and glycoconjugates. This methodology has only recently been pursued. Wong and coworkers [51] described the first example of whole cell glycosylation with a strain of *E. coli.* containing a recombinant α-1,2-manno-

Figure 4



Glycolipid acceptors for the oligosaccharyltransferase. Only the natural lipid moiety (dolichyl) displays significant activity [46].

Figure 5



Synthesis of a novel glycoprotein using glycosyltransferases and a peptide ligase. A monoglycosylated form of RNase B can be prepared either by deglycosylation of native RNase B or by ligation of peptide fragments. This can be further glycosylated to give a protein-linked tetrasaccharide. Adapted with permission from [48••].

syltransferase from *Saccharomyces cerevisiae* [51]. Since then, there have been reports of whole cell glycosylation by *S. cerevisiae* harbouring recombinant α-2,6-sialyltransferase [52] and β-1,4-galactosyltansferase [53]. These results demonstrate that transformed whole cells can be used as a convenient biocatalyst for glycosylation.

Conclusions

Enzymes have been shown to be useful tools for the synthesis of oligosaccharides and glycoconjugates on a reasonable timescale. Reactions have been scaled up to generate gram quantities of product, which would be difficult to achieve by multistep chemical synthesis. Glycosidases have proven to be useful for the synthesis of di- and trisaccharides, utilising relatively simple starting materials, while Endo-A has been used for the preparation of neoglycoconjugates containing high-mannose oligosaccharides.

Studies with analogues have shown that both glycosidases and glycosyltransferases tolerate some modifications, in particular in glycosyl acceptor structure, so that they may also be used in the synthesis of unnatural oligosaccharide analogues. Mechanistic studies have been performed on a fucosyltransferase, and structure/activity relationships have been obtained for other glycosyltransferases. Both glycosidases and glycosyltransferases are sure to become ever more useful as practical synthetic tools for the synthesis of complex oligosaccharides and glycoconjugates. Glycosyltransferases are starting to show promise as reagents for solid-phase synthesis, and our knowledge of the oligosaccharyltransferase continues to grow.

Exciting progress has been made in the synthesis of glycoproteins and it seems likely that the total synthesis of novel, designed glycoproteins will soon be possible. The main limitation, in particular for glycosyltransferases, is the availability of enzyme. This may be overcome by the use of whole cells of yeast or bacteria containing recombinant glycosyltransferases, and significant progress is being made in this area.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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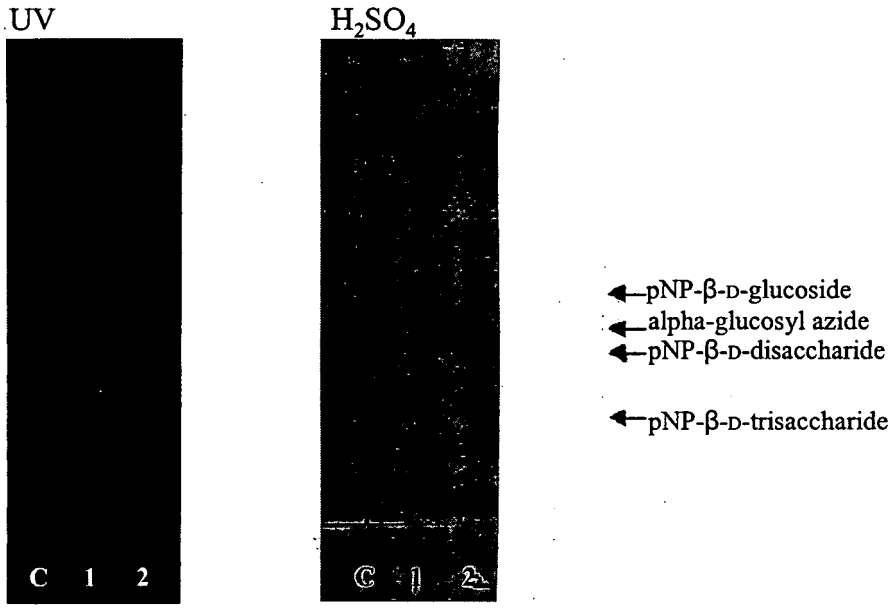
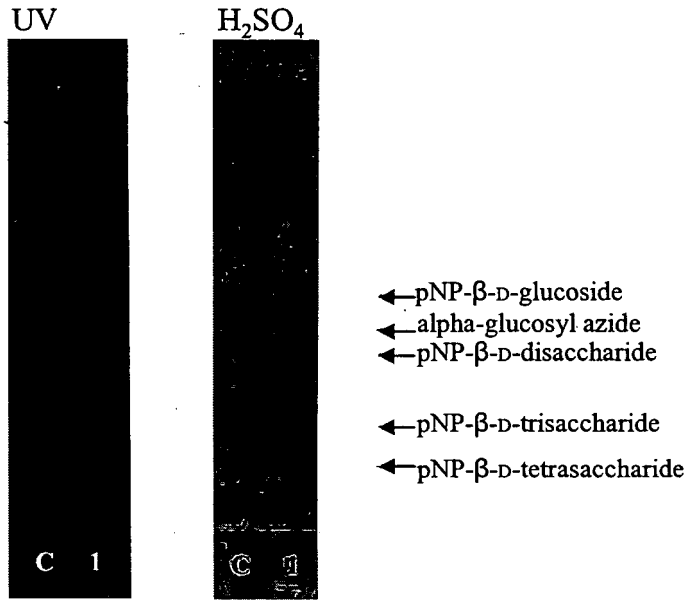


C : 25 mM α-glucosyl azide, 12.5 mM pNP-β-D-glucoside

1 : substrate + AbgE358G (2.5 mg/ml)

2 : substrate + AbgE358G (5.0 mg/ml)

substrate : 25 mM α-glucosyl azide, 12.5 mM pNP-β-D-glucoside



C : 25 mM α-glucosyl azide, 12.5 mM pNP-β-D-glucoside

1 : substrate + AbgE358G (5.0 mg/ml)

substrate : 100 mM α-glucosyl azide, 25 mM pNP-β-D-glucoside